OROBOROS INSTRUMENTS
high-resolution respirometry

Protocols

Mitochondrial Physiology Network 03.02: 1-8 (2013)
©1998-2013 OROBOROS®
Version 15: 2013-10-08

Selected media and chemicals for respirometry with mitochondrial preparations

Fontana-Ayoub M, Eigentler A, Fasching M, Gnaiger E

OROBOROS INSTRUMENTS Corp., high-resolution respirometry
Schöpfstr. 18, A-6020 Innsbruck, Austria
erich.gnaiger@oroboros.at; www.oroboros.at

Summary: Different media for tissue preparation and respiration are used in investigations of mitochondrial function. Initial decisions on the composition of media and chemicals are decisive for long-term studies and crucial for comparability of results. As a guideline, we summarize an update of our experience with media and chemicals for high-resolution respirometry with isolated mitochondria, permeabilized cells, muscle fibres and tissue homogenates. Whereas optimization is necessary for specific experimental protocols, standardization will improve the comparability of data obtained in different laboratories. Such efforts towards standardization are important for the advancement of mitochondrial physiology and mitochondrial medicine.

Section

1. Introduction

High-resolution respirometry provides the basis for a detailed analysis of mitochondrial function. Incubation media must be chosen which contain compounds such as sucrose, mannitol, potassium chloride, potassium-MES, to achieve physiological osmolarity. Additional components are added to preserve mitochondrial integrity. Mitochondrial media, therefore, have different ionic strengths, pH and ionic compositions.

The list of **media** is organized according to the major applications, including the isolation of mitochondria, the preparation of muscle fibres, and the incubation media for respirometry, with emphasis on **MiR06** (MiR06 = MiR05+Catalase; MiPNet14.13); as our most advanced respiration medium. The list of **chemicals** contains mitochondrial substrates, inhibitors, uncouplers and agents for cell permeabilization. The preferred concentrations and solvents are shown for stock solutions, and storage conditions are recommended.

Finding a compromise between dynamic optimization of experimental protocols and adherence to a fixed standard represents a well-known problem in the development and application of strategies for scientific investigation. Improvement of standard methods requires cooperation and feedback. Therefore we appreciate any comments and suggestions directed towards improved and more generally acceptable standards for studies of mitochondrial function.

2. Media for muscle fibre preparation and isolation of mitochondria

Higher respiratory capacities are observed when integrating a preservation strategy in the formulation of isolation media (such as addition of antioxidants). Improvement of the quality of isolation media may be limited by the increasing cost when preparing large volumes. The media for isolation of mitochondria (Section 2.2 and 2.3) are minimum media without concerns on preservation strategies.

2.1. Preparation of permeabilized muscle fibres - BIOPS

(Veksler et al 1987; Letellier et al 1992)

The relaxing and biopsy preservation solution BIOPS contains 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1.

BIOPS Total volume = 1 litre

	Final conc.	FW	Stock solution	Addition to 1 litre final	Source and product code
CaK_2EGTA*	2.77 mM		100 mM	27.7 ml	
K_2EGTA*	7.23 mM		100 mM	72.3 ml	
Na_2ATP	5.77 mM	551.1		3.141 g	Sigma, A 2383 (5g)
$MgCl_2 \cdot 6\ H_2O$	6.56 mM	203.3		1.334 g	Scharlau, MA 0036 (250g)

Taurine	20 mM	125.1		2.502 g	Sigma, T 0625 (25 g)
Na_2Phosphocreatine	15 mM	255.8		4.097 g	Sigma, P 7936 (5 g)
Imidazole	20 mM	68.1		1.362 g	Fluka, 56750 (100 g)
Dithiothreitol (DTT)	0.5 mM	154.2		0.077 g	Sigma, D 0632, (1g)
MES	50 mM	195.2		9.76 g	Sigma, M8250 (250 g)

BIOPS contains the following ion concentrations:

Ca^{2+} free	0.1 µM
Mg^{2+} free	1 mM
MgATP	5 mM
Ionic strength	160 mM

Adjust the pH to 7.1 (with 5 N KOH) at 0 °C. Divide into 20 ml portions. Store BIOPS and K_2EGTA / CaK_2EGTA solutions frozen at -20 °C in plastic vials.

* Anhydrous; preparation of stock solutions K_2EGTA and CaK_2EGTA:

K_2EGTA Mix 100 mM EGTA (Sigma, E 4378, 25 g) and 200 mM KOH (Sigma, P 1767, 1 kg) (dissolve 7.608 g EGTA and 2.3 g KOH in 200 ml H_2O, adjust the pH to c. 7.0 with KOH).

CaK_2EGTA Dissolve 2.002 g $CaCO_3$ (Sigma, C 4830; 100g) in 100 mM hot (80 °C) solution of EGTA (7.608 g / 200 ml) while stirring continuously, add 2.3 g KOH, adjust the pH to c. 7.0.

KH_2PO_4 ATP will be hydrolyzed at least partially during fibre storage, thus generating mM levels of inorganic phosphate. It has not been reported if addition of 3 mM phosphate (Veksler et al 1987; Skladal et al 1994) exerts any effect on preservation quality.

Saponin solution: for muscle permeabilization, prepare everyday new:

1. For saponin stock solution, add 5 mg of saponin (Sigma, S 2149; 25 g) to 1 ml BIOPS.
2. For permeabilization in saponin solution, add 21 µl of the saponin stock solution to 2 ml of BIOPS.

2.2. Isolation of mitochondria from liver, heart, placenta

Medium A1: total volume 1 litre

	Final conc.	FW	Addition to 1 litre final volume
Sucrose	250 mM	342.3	85.6 g
Na_2EDTA	0.5 mM	372.2	0.186 g
Tris	10 mM	121.1	1.211 g

Adjust the pH to 7.4 (HCl) at c. 0 °C. Store frozen at -20 °C in 100-200 ml plastic vials.

Medium B1: take 500 ml of medium A1 and add:

BSA	1 g/l		0.5 g/500 ml

Store frozen at -20°C in 100-200 ml plastic vials.

2.3. Isolation of mitochondria from skeletal muscle

Medium A2: total volume 1 litre

	Final conc.	FW	Addition to 1 litre final volume
KCl	180 mM	74.55	13.42 g
Na_2EDTA	0.5 mM	372.2	0.186 g
Tris	10 mM	121.1	1.211 g

Adjust the pH to 7.4 (HCl) at c. 0 °C. Store frozen at -20 °C in plastic vials.

Medium B2: take 500 ml of medium A2 and add:

BSA	1 g/l		0.5 g/500 ml

Store frozen at -20 °C in plastic vials.

3. Mitochondrial respiration media (MiR)

3.1. MiR06

MiR05: Isolated mitochondria (liver and cardiac), and permeabilized endothelial cells (see Gnaiger et al 2000).

Preparation of **MiR06** (= MiR05 + Catalase): see separate Protocol (MiPNet14.13).

3.2. Oxygraph medium for cytochrome *c* test

The high concentration of KCl favours dissociation of cytochrome *c* from the inner mitochondrial membrane and cytochrome *c* release upon injury of the outer mitochondrial membrane. Respiratory flux is reduced with cytochrome *c* depletion, and can be restored after addition of 10 µM cytochrome *c* (Saks et al 1992, 1995; Gnaiger and Kuznetsov 2002; Kuznetsov et al 2004).

	Final conc.	FW	Addition to 1 litre final
EGTA	0.4 mM	336.2	0.134 g
$MgCl_2.6\ H_2O$	3 mM	203.3	0.61 g
KH_2PO_4	5 mM	136.1	0.68 g
Dithiothreitol	0.3 mM	154.2	0.046 g
KCl	125 mM	74.55	9.32 g
HEPES	20 mM	238.3	4.77 g

Cytochrome *c* medium contains the following ion concentrations:

Ca^{2+} free	0.0 µM
Mg^{2+} free	2.51 mM
EGTA free	0.36 µM
Ionic strength	142 mM

Adjust the pH to 7.1 (5 N KOH) at 25 °C. Divide into 20 ml portions. Store frozen at -20 °C in plastic vials.

4. Chemicals for the study of mitochondrial and cellular bioenergetics

Calculation of concentrations: MiPNet09.12_O2k-Titrations.xls

4.1. Mitochondrial substrates

Substrate	FW	Stock soln. Conc [mM]	Stock soln. Amount	Comments	Source, product code and storage
G: L-Glutamic acid, sodium salt, $C_5H_8NO_4Na$ (contains 1 mol/mol H_2O)	187.1 169.1 anhy-drous	2000	3.742 g/ 10 ml H_2O	Neutralize with 5 N KOH, check pH. Divide into 0.5 ml portions. Store frozen at -20 °C.	Sigma, G 1626 (100g) R.T
M: L-Malic acid, $C_4H_6O_5$	134.1	800	1.073 g/ 10 ml H_2O	Neutralize with 10 N KOH, check pH. Divide into 0.5 ml portions. Store frozen at -20 °C.	Sigma, M 1000 (100 g) R.T
P: Pyruvic acid sodium salt, $C_3H_3O_3Na$	110.0	2000	44 mg/ 0.2 ml H_2O	Prepare everyday new.	Sigma, P 2256 (25 g); 4°C
S: Succinate disodium salt, hexahydrate, $C_4H_4O_4Na_2$. 6 H_2O	270.1	1000	2.701 g/ 10 ml H_2O	Adjust pH to 7.0 with 37% HCl. Divide into 0.5 ml portions. Store frozen at -20 °C.	Sigma, S 2378 (100 g) R.T
Oct: DL-Octanoyl-carnitine-HCl, $C_{15}H_{30}NO_4Cl$	323.85	100	32.4 mg/ 1 ml H_2O	Store frozen at -20 °C.	TOCRIS Bioscience, No. 0605 (50 mg), RT, desiccate
Pal: Palmitoyl-DL-carnitine-HCl, $C_{23}H_{45}NO_4 \cdot HCl$	436.1	10	8.72 mg/ 2 ml H_2O	Store frozen at -20 °C.	Sigma P 4509 (100 mg) -20 °C
As: Ascorbate sodium salt, $C_6H_7O_6Na$	198.1	800	1.584 g/ 10 ml H_2O	To prevent autooxidation, adjust pH to ca 6 with ascorbic acid (a 137.6 mg ml^{-1} solution of pH ca 2). Divide into 0.2 ml portions. Store frozen at -20 °C. Light sensitive.	Sigma, A 4034 (100 g) R.T
Tm: TMPD *N,N,N',N'*-Tetramethyl-p-phenylenediamine dihydrochloride, $C_{10}H_{16}N_2 \cdot 2$ HCl	237.2	200	47.4 mg/ 1 ml H_2O	To prevent autooxidation add 0.8 M ascorbate to a final concentration of 10 mM. Divide into 0.2 ml portions. Store frozen at -20 °C.	Sigma T 3134 (5 g) R.T
c: Cytochrome *c*	12500	4.0	50 mg/ 1 ml H_2O	Divide into 0.2 ml portions. Store frozen at -20 °C.	Sigma, C 7752 (50 mg) -20 °C

D: ADP** (Adenosine 5'diphosphate, $C_{10}H_{15}N_5O_{10}P_2K$, potassium salt, contains 1 mol/mol H_2O)	501.3	500	0.501 g/ 2 ml H_2O	Neutralize with 5 N KOH (approx.450 µl), check pH. Divide into 0.2 ml portions. Store frozen at -80 °C.	Calbiochem, 117105 (1 g) 4°C or Sigma, A 5285 (1 g) -20 °C
T: ATP** (Adenosine 5'-triphosphate, $C_{10}H_{14}N_5O_{13}P_3Na_2$, disodium salt, contains 3.5 mol/mol H_2O)	614.1 3.5 mol/ mol H_2O 551.1 anhy-drous	500	0.614 g/ 2 ml H_2O	Neutralize with 5 N KOH (approx. 400 µl), check pH. Divide into 0.2 ml portions. Store frozen at -80 °C.	Calbiochem, 1191 or Sigma, A 2383 (5 g) or -20 °C

** To keep $[Mg^{2+}]$ constant, add $MgCl_2$ (0.6 mol/mol ADP or 0.8 mol/mol ATP).

4.2. Mitochondrial inhibitors

Inhibitor	FW	Stock soln. Conc. [mM]	Stock soln. Amount	Comments	Source, product code and storage
Ama: Antimycin A	540	5.0	11 mg/ 4 ml ethanol	Divide into 0.2 ml portions. Store at -20 °C. Very toxic.	Sigma A 8674 (25 mg) -20 °C
Amy: Amytal (Amobarbital) sodium salt, $C_{11}H_{17}N_2O_3Na$	248.3	200	0.497 g/ 10 ml 50% ethanol	Divide into 0.5 ml portions. Store frozen at -20 °C. Light sensitive. Toxic.	
Atr: Atractyloside dipotassium salt, $C_{30}H_{44}O_{16}S_2K_2$ (2.5 mol/mol H_2O)	803.0	50	40.2 mg/ 1 ml H_2O	Dissolves better in warm water. Store frozen at -20 °C. Toxic.	Sigma A 6882 (250 mg) R.T.
Azd: Sodium azide, NaN_3	65.01	4000	260 mg/ 1 ml H_2O	Divide into 0.5 ml portions. Store frozen at –20 °C. Very toxic.	Sigma S 2002 (25 g) R.T.
Cat: Carboxy-atractyloside, potassium salt	802.99 free acid	5.0	4.02 mg/ 1 ml H_2O	Divide into 0.2 ml portions. Store at -20 °C. Toxic.	Sigma C 4992 (2 mg) -20°C
Kcn: Potassium cyanide, KCN	65.12	1000	13 mg/ 0.2 ml H_2O	Prepare everyday new. The pH of the solution may be very alkaline; adjust with HCl. Photosensitive. Hygroscopic. Very toxic.	Fluka 60178 (100 g)
Mna: Malonic acid	104.06	2000	0.0208 g/ 100 µl	Dissolve in 35 µl H_2O+65 µl of KOH 5 N, check pH, prepare fresh	Sigma Aldrich M129-6 (5 g) R.T.
Myx: Myxothiazol	487.7	1.0	1.0 mg/ 2.05 ml ethanol	Divide into 0.2 ml portions. Store at -20 °C. Very toxic.	Sigma T-5580 (1mg) 4°C

Omy: Oligomycin	800	4 mg/ml	4 mg/ 1 ml ethanol	Divide into 0.2 ml portions. Store at -20 °C. Very toxic.	Sigma O 4876 (5 mg) -20 °C
Oua: Ouabain (G-Strophanthin) octahydrate, $C_{29}H_{44}O_{12}.8\ H_2O$	728.8	10	7.3 mg/ 1 ml H_2O	Divide into 0.2 ml portions. Store at -20 °C. Light sensitive. Toxic.	
Pep: p5-Di (adenosine -5') penta-phosphate sodium salt, $C_{20}H_{29}N_{10}O_{22}P_5$ (5 mol/mol Na, 1.5 mol/mol H_2O)	1058.4 916.4 free acid	50	52.91 mg/ 1 ml H_2O	Neutralize with 5 N KOH, check pH. Divide into 0.2 ml portions. Store at -20 °C. Toxic.	
Rot: Rotenone, $C_{23}H_{22}O_6$	394.4	1.0 [a]	3.94 mg/ 10 ml ethanol	Difficult to dissolve. Store at -20 °C. Light sensitive. Very toxic.	Sigma R 8875 (1 g) R.T.
Rut: Ruthenium red (ammoniated ruthenium oxychloride)	551.22	10	5.5 mg/ 1 ml H_2O	Store frozen at -20 °C.	

[a] Rotenone is added at a high final concentration (0.5 µM), based on a 1.0 mM stock solution. Since 0.1 µM may be fully inhibiting some mitochondrial preparations, a lower concentration may be used (0.2 mM stock, 0.1 µM final), to reduce the problem of rotenone retention in the O2k-chamber.

4.3. Mitochondrial uncouplers (protonophores)

Uncoupler	FW	Stock soln. Conc. [mM]	Stock soln. Amount	Comments	Source, product code and storage
DNP: 2,4-Dinitrophenol, $C_6H_4O_5N_2$	184.1	10	3.7 mg/ 2 ml H_2O	Neutralize with 1 N KOH, check pH. Store frozen at –20 °C. Toxic.	
F (FCCP): Carbonyl cyanide p-(trifluoro-methoxy) phenyl-hydrazone $C_{10}H_5F_3N_4O$	254.2	1.0	2.54 mg/ 10 ml ethanol	Divide into 0.5 ml portions. Store in glass vials at -20 °C.	Sigma C 2920 (10 mg) 4 °C
TTFB: 4,5,6,7-Tetrachloro-2-trifluoromethyl-benzimidazole	323.94	1.0	3.24 mg/ 10 ml ethanol	Divide into 0.5 ml portions. Store at -20 °C.	

4.4. Agents for cell permeabilization

Substance	FW	Stock sol. Conc.	Stock solution Amount	Comments	Source, product code and storage

Dig: Digitonin	1229.3	8.1 mM	10 mg/1 ml DMSO	Store frozen at -20 °C. Toxic.	Fluka 37008 (1 g) RT
Sap: Saponin	-	5 mg/ml	5 mg/1 ml H_2O	Prepare everyday new.	Sigma S 2149 (25 g)

5. General comments

5.1. Solutions stored at low temperature: Mix carefully after re-warming, since phase separation may occur and compounds may precipitate in cold solutions. During the course of the experiment, keep stock solutions on ice.

5.2. Solutions contain ethanol: there may be a problem of evaporation and subsequent increase of concentration of stock solutions.

5.3. Chemicals dissolved in ethanol or DMSO: To check the influence of ethanol or DMSO on mitochondrial function and experimental sensors (ion selective electrodes), the same additions of pure solvents should be used in control experiments.

5.4. For all stock solutions of mitochondrial substrates, inhibitors, and uncouplers; the total volumes of solutions are indicated.

5.5. Store chemicals as indicated by the suppliers. The storage conditions of prepared solutions are indicated in the comments.

6. References

Gnaiger E, Kuznetsov AV (2002) Mitochondrial respiration at low levels of oxygen and cytochrome *c*. Biochem Soc Trans 30: 242-248.

Gnaiger E, Kuznetsov AV, Schneeberger S, Seiler R, Brandacher G, Steurer W, Margreiter R (2000) Mitochondria in the cold. In: *Life in the Cold.* (Heldmaier G, Klingenspor M, eds) Springer, Heidelberg, Berlin, New York: pp 431-442.

Letellier T, Malgat M, Coquet M, Moretto B, Parrot-Roulaud F, Mazat J-P (1992) Mitochondrial myopathy studies on permeabilized muscle fibres. Pediatr Res 32: 17-22.

Kuznetsov AV, Schneeberger S, Seiler R, Brandacher G, Mark W, Steurer W, Saks V, Usson Y, Margreiter R, Gnaiger E (2004) Mitochondrial defects and heterogeneous cytochrome c release after cardiac cold ischemia and reperfusion. Am J Physiol Heart Circ Physiol 286: H1633–H1641.

Pesta D, Gnaiger E (2012) High-resolution respirometry. OXPHOS protocols for human cells and permeabilized fibres from small biopisies of human muscle. Methods Mol Biol 810: 25-58.

Saks VA, Belikova YuO, Vasilleva EV, Kuznetsov AV, Lyapina S, Petrova L, Perov NA (1993) Retarded diffusion of ADP in cardiomyocytes: possible role of mitochondrial outer membrane and creatine kinase in cellular regulation of oxidative phosphorylation. Biochim Biophys Acta 1144: 134-148.

Saks VA, Kuznetsov AV, Khuchua ZA, Vasileva EV, Belikova YO, Kesvatera T, Tiivel T (1995) Control of cellular respiration by mitochondrial outer membrane and by creatine kinase in normal muscle and in pathology. J Mol Cell Cardiol 27: 625-645.

Skladal D, Sperl W, Schranzhofer R, Krismer M, Gnaiger E, Margreiter R, Gellerich FN (1994) Preservation of mitochondrial functions in human skeletal muscle during storage in high energy preservation solution (HEPS). In: *What is Controlling Life?* (Gnaiger E, Gellerich FN, Wyss M, eds) Modern Trends in BioThermoKinetics 3 Innsbruck Univ. Press: 268-271.

Veksler VI, Kuznetsov AV, Sharov VG, Kapelko VI, Saks A (1987) Mitochondrial respiratory parameters in cardiac tissue: a novel method of assessment by using saponin-skinned fibres. Biochim Biophys Acta 892: 191-196.